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1. Your reference

SPG/P100404GB

2. Patent application number
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3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Keele University
Keele
Staffordshire
ST5 5BG
GB

Patents ADP number *(if you know it)*

6014630002

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention

Method

5. Name of your agent *(if you have one)*

Harrison Goddard Foote

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

31 St Saviourgate
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6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
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Continuation sheets of this form	
Description	9
Claim *(s)*	6
Abstract	-
Drawing *(s)*	1 + 1

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Request for substantive examination *(Patents Form 10/77)*	
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11. I/We request the grant of a patent on the basis of this application.

Signature S P Gilholm Date 18 June 2002

12. Name and daytime telephone number of person to contact in the United Kingdom S P Gilholm 01904 732120

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Method

This invention relates to a novel method of magnetically manipulating cells *in vivo* and to methods of treatment related thereto.

It is well established in pharmacology that communication, e.g. between cells, is governed by ion channels within the cells. A wide variety of such channels exist, for example potassium, calcium and sodium channels. Pharmaceutically active chemical compounds are often used to block such channels resulting in a pharmacological effect. For example, calcium antagonists are known to be active on the cardiovascular system, for example by reducing the magnitude of the calcium current in the sino-atrial and atrio ventricular nodes. An important aspect of ion channel control is determining when the channel opens (gating).

Ion channels generally possess ionic selectivity which is an extremely important aspect of the channel's functional properties. Channels are generally characterised by their ionic selectivity, for example

- sodium channel
- potassium channel
- calcium channel
- chloride channel
- non-selective cation channel.

Ion channels are large integral membrane proteins that form pores through a cellular plasma membrane allowing ions to cross by flowing down an electrochemical gradient through the channels (passive transport). The core of the pore is generally hydrophilic, and contains a part of the protein which recognises only certain ions thus acting as a selectivity filter. Gates in the channel can open in response to a variety of stimuli, including changes in membrane potential, mechanical activation or the presence of certain chemicals outside or inside the cell. More than 50 types of ion channels have been identified.

Ca channels, like Na channels, are voltage-gated, open when the internal voltage becomes more positive than the resting potential, and inactivate, or close, spontaneously even though the voltage stimulus is maintained. Ca channels are effective in the axon terminals of neurons, and in invertebrate muscle, vertebrate smooth muscle, and participate with Na channels in vertebrate cardiac muscle. Ca++ channels participate in action potentials when you need to get Ca++ into the cell to do something, such as make cardiac muscle contract, or release neurotransmitter at the axon terminal.

Na channels are almost all voltage-gated, that is, their gates open in response to changes in membrane potential, usually when the inside of the cell becomes more positive. Most Na channels are closed, or inactivate, spontaneously in a few milliseconds even though the membrane potential remains at the level which opened them. Na channels are found in neurons, vertebrate skeletal muscle, and cardiac muscle. Na serves to let charge into the cell; the Na itself doesn't do anything chemically.

Potassium channels, like Na channels, tend to be voltage-gated and to open when the inside of the cells becomes more positive. They mostly open at voltage more positive than Na or Ca channels, and most of them stay open as long as the voltage stays positive. Since the Nernst potential for K is near -80mV, opening K channels at voltages near +20mV lets K out and makes the internal voltage more negative. This in turn closes the K channels. This is how the action potential repolarises, or returns to resting potential. There are also K channels that are not voltage-gated. These are open at the resting potential, and in fact set the resting potential.

As yet the mechanism by which these channels are activated is not known and there is no knowledge of how to selectively target specific membrane channels for mechanical activation.

International Patent Application No. WO 01/88540 describes silica coated nanoparticles which comprise a magnetic metal core. The magnetic core present in the nanoparticles enables the particles to be responsive to a magnetic field and therefore, the particles are suitable for use in diagnostic, imaging and recording systems. However, the nanoparticles of the prior art may suffer from the disadvantage that they do not define the method of activation at a cellular level.

Magnetic bead twisting cytometry has been used to define the mechanical properties of single cells and to demonstrate that external mechanical forces can be transmitted across the cell surface and through the cytoskeleton via transmembrane cell adhesion molecules such as integrins.

We have now found a method of selectively activating cells which enables the cells to then be manipulated mechanically in a remote manner, e.g. from outside the body.

Thus according to the invention we provide a method of magnetically manipulating cells *in vivo* which comprises the association of a magnetisable particle with a cell.

More particularly, the invention provides a method of agonising or antagonising ion channels within a cell which comprises the association of a magnetisable particle with a cell as hereinbefore described.

The association of a magnetisable particle with a cell may comprise the introduction of such a particle into a cell, the attachment of such a particle to a cell, e.g. externally or internally to a cell, or any combination thereof.

Preferentially, the method of the invention comprises the remote manipulation of cells and/or of agonising or autagonising ion channels, e.g. manipulation from outside the body.

The method of the invention may be utilised in relation to a variety of cells which are known *per se*. However, preferentially, the method is suitable for use with mammalian somatic cells, for example, bone, cartilage, muscle (skeletal and cardiac) lymphatic cells, endocrine cells, urinary system cells, cells relating to the reproduction system, and neuronal cells.

The method of the invention may be utilised in connection with any conventionally known ion channels within the cell which are hereinbefore described. The method is especially suited for use in mechanosensitive ion channels. Such mechanosensitive ion channels have been identified in many cell types and have been predominantly described as calcium or potassium ion channels, although it should be understood that the method of the invention is not limited to use in relation to calcium or potassium ion channels. By way of example only, one such channel which has been well characterised at the molecular level and at the functional level in neuronal cells is the chromosomal gene TREK-1, which is part of the 2P K+ channel family. TREK-1 channels, have been identified in bone cells, and are known to respond to shear stress, cell swelling and membrane stretch as well as other external agents such as fatty acids and general anaesthetics.

A particular aspect of the present invention is to provide a method of manipulating mechanosensitive ion channels.

These "mechanosensitive" ion channels are present in a variety of mammalian, e.g. human, cells and the present invention enables the cells to be selectively activated in the body and/or in cell cultures. As these channels are instrumental in normal cellular function and play a particularly important role in, for example, the production of bone and connective tissue or activation of the peripheral nervous system, the ability to manipulate them remotely, e.g. from outside the body, is especially advantageous an provides applications in, *inter alia*, pain relief, e.g. anaesthetics, therapeutics, tissue engineering and repair.

In a further aspect of the invention the method may also be suitable for use with conventionally non mechanosensitive cells and/or ion channels by the transfection of channels into cells which may otherwise be otherwise non-responsive.

A wide variety of particles may be used in the method of the invention. Generally, any magnetic material may be used, examples of which include magnetite (Fe_3O_4), maghemite (γFe_2O_3), chromium oxide (CrO_2) and greigite (Fe_3S_4). Preferably the magnetic material comprises nanoparticles which comprises a magnetic core with a biocompatible coating. Thus, such preferred particles are nanoparticles having a core and, e.g. a silica shell enveloping the core. However, also porous particles with multiple magnetic centres within the pores. An example of such nanoparticles are those described in International Patent application No. WO 01/88540 which is incorporated herein by reference.

The micro- and nano- particles (intended to be attached to the cells) may will generally be substantially spherical or elliptical. The size of the nanoparticles may vary according, *inter alia*, to the nature of the magnetic material, the application, etc. However, an example of nanoparticles may be nanoparticles can having a mean size, e.g. diameter, of 5000 nm or less, e.g. from 1 nm to 5000 nm, preferably from 1 nm to 1000 nm, more preferably from 1 nm to 300 nm, or from 2 nm to 10 nm).

The particles for attachment to the cells may be coated or uncoated and single or multi-domain. Examples of suitable particles include, but are not limited to:

(i) Coated magnetic microspheres (d = 4 μm) available from Spherotech, Inc. These microspheres consist of a magnetically blocked core - coated by a polymer.

(ii) Single-domain, ferrite-doped silica nanoparticles with tunable size (d = 50-300 nm) and narrow size distribution.

In the method of the invention the ion channels may be activated by attaching the magnetic particles as hereinbefore described to specific regions of the cellular membrane and/or to specific "receptors" on the ion channels themselves. Thus, the mechanical forces required to activate the channels can then be applied remotely by a magnetic field acting on these magnetic particles.

In particular the method of the invention comprises modifying a magnetic nanoparticle as hereinbefore described by tagging the nanoparticle with one or more specific antibodies or protein binding motifs which recognise key cellular elements within a cell. These include transmembrane adhesion molecules, such as integrins, cadherins, selectins, and immunoglobulins or dispersed membrane adhesion proteins such as RGD (arginine-glycine-aspartate).

The method of the invention is especially advantageous because it provides a method of treatment of a variety of disorders. Indeed the invention provides a method of treatment which is applicable to any disorder in which one or more ion channels play a role. In addition, the invention provides a method for potential control of ion channel activation including pain relief, e.g. an anaesthetic role.

Thus according to the invention we provide a method of treatment of a patient suffering from a disorder in which an ion channel plays a role which comprises the administration to such a patient of magnetic nanoparticles as hereinbefore described and manipulating those particles using a magnetic field.

The method of treatment as hereinbefore described should not be considered to be limited, but it is especially advantageous in tissue and/or bone repair. The method of treatment can be to facilitate further treatment by providing a method of pain relief, e.g. for localised anaesthesia, to targeted regions of the body.

The nature of such cells may vary depending upon the nature of the tissue of interest. For example, the cells may be ligamentum cells for growing new ligaments,

tenocytes for growing new tendon. Alternatively, the cells may be chondrocytes and/or other stromal cells, such as chondrocyte progenitor cells.

Thus the method of the invention may include the regeneration of tissue or the generation of artificial tissue, such as skin, cartilage, ligament, tendon, muscle or bone.

Alternatively the method may comprise wound healing and/or tissue adhesion.

In a preferred embodiment the method may comprise bone repair and/or bone growth.

In a yet further alternative the method of the invention may include, for example, dental applications and/or veterinary applications.

In the method of the invention the magnetic field may be varied depending upon, *inter alia*, the nature of the disorder to be treated, but may, for example, be at a frequency of from 0.1 to 10 Hz. But, frequencies outside this range can also be used. The magnetic field will typically have a flux density in the order of (but not limited to) 10 mT to 1400 mT.

In the method of the invention the magnetic field may be generated outside the body for the case of *in vivo* applications, and may be provided by a permanent magnet or an electromagnet. The magnetic field may be a constant or a variable field, e.g. a permanent magnet may be moved relative to the cells. In the case of an electromagnet, a magnetic field may be generated by provision of appropriate electric current levels to the electromagnetic, optionally, in combination with alternating current.

According to a yet further aspect of the invention we also provide the use of a magnetic nanoparticle in a method of magnetically manipulating cells *in vivo*. In a

preferred embodiment we provide the use as wherein the use comprises tissue and/or bone repair as hereinbefore described.

The invention will now be described by way of example only and with reference to the accompanying drawings in which Figure 1a) is a schematic representation of the structure of TREK-1 showing the three sites of 12x histidine insertions for tagging magnetic beads for mechanical manipulation; and

Figure 1b) illustrates primary human astrocytes with membrane bound RGD coated carboxyl ferromagnetic particles (4μm) (magnification x 1000).

Example 1

Activation of TREK-1 using magnetic cytometry

The modified TREK-1 gene was transvected into the human HEK 293 cell line. Detection of plasmid transfection efficiency was conducted by monitoring CD8 expression using immunocytochemsitry, electrophysiology using whole cell recordings and a fluorescent marker for membrane depolarisation monitored via confocal microscopy. Three regions of the molecule were tagged for experimental manipulation by insertion for a 12x histidine coding sequence as shown in Figure 1a. Functionalised, magnetic micro- and nano-particles were coupled to the cell membrane using the 12x His antibody coatings to enable force to be applied to different regions of the channel. Particles were twisted by moving high-field rare earth magnets (NdFeB) magnets with surface flux density of up to 1.4 Tesla, about a vertical axis near the cell culture. During this process, cells were monitored via phase contrast microscopy and confocal microscopy. Electrical activity in cells were monitored using whole cell recordings.

Specific monoclonal antibodies raised to the three regions prior to histidine insertions outlined above have been raised for tagging endogenous TREK channels in vivo.

Example 2

Non-specific membrane deformation using magnetic cytometry

Biocompatible magnetic micro- and nanoparticles were coupled to the cell membrane (specifically with monoclonal antibodies to RGD containing peptides and collagen) to stretch generalised regions of the cell (Figure 1b). The torque applied to magnetically blocked particles deforms the cell membrane and activates nearby MS ion channels following application of a range of magnetic fields. In addition, the cells were biochemically assayed to determine whether reaction pathways are being initiated by magnetic twisting (e.g. prostaglandin and extracellular matrix production). MS ion channel blockers such as gadolinium or amiloride were added extracellularly to confirm whether the MS channels are instrumental in any observed changes.

Initial experiments employed functionalised magnetic microspheres (d=4 μm) available from Spherotech. Inc. In addition magnetically blocked, ferrite-doped silica nanoparticles with tunable size (d=50-300nm) and narrow size distribution and PVA/magnetite nanoparticle-based ferrofluids (d=4-10nm) were synthesised. High-field rare earth magnets again were used to generate the applied fields.

Example 3

Magnetic activation in a 3D model

The use of magnetic strategies for spatially targeted ion channel activation in a 3D, cell-seeded scaffold was investigated by applying a magnetic field across a cell-seeded construct within a bioreactor. The ion channels in the cells were activated within the scaffold and the long-term effects of this ion channel activation on matrix synthesis and cell proliferation assessed. Magnetic particle-based approaches with a non-specific activation and a TREK-transfected bone and cartilage cell lined model were used.

Claims

1. A method of magnetically manipulating cells *in vivo* which comprises the association of a magnetisable particle with a cell.

2. A method according to claim 1 characterised in that the method comprises agonising or antagonising ion channels within a cell which by the association of a magnetisable particle with a cell.

3. A method according to claim 1 characterised in that the method comprises the remote manipulation of cells.

4. A method according to claim 1 characterised in that the cells are mammalian cells.

5. A method according to claim 4 characterised in that the cells are derived from connective or neuronal tissue.

6. A method according to claim 5 characterised in that the cells are derived from bone, neurons, cardiac cells etc.

7. A method according to claim 2 characterised in that the ion channel is a mechanosensitive ion channel.

8. A method according to claim 7 characterised in that the mechanosensitive ion channel has been transfected into a cell.

9. A method according to claim 2 characterised in that the ion channel is selected from the group including sodium channel, potassium channel, calcium channel, chloride channel and a non-selective cation channel.

10. A method according to claim 9 characterised in that the ion channel is selected from a calcium or a potassium ion channel.

11. A method according to claim 9 characterised in that the ion channel is a potassium ion channel.

12. A method according to claim 10 characterised in that the potassium channel is a TREK-1 channel.

13. A method of manipulating a mechanosensitive ion channel characterised in that the method comprises the association of a magnetisable particle with an ion channel.

14. A method according to claim 1 or 13 characterised in that the magnetic material is selected from the group which includes magnetite (Fe_3O_4), maghemite (γFe_2O_3), chromium oxide (CrO_2) and greigite (Fe_3S_4).

15. A method according to claim 1 or 13 characterised in that the magnetic material comprises nanoparticles which comprises a magnetic core with a biocompatible coating.

16. A method according to claim 15 characterised in that the nanoparticle has a core and a silica shell enveloping the core.

17. A method according to claim 16 characterised in that the nanoparticle is selected from those described in International Patent application No. WO 01/88540.

18. A method according to claim 13 characterised in that the particle is a porous particle with multiple magnetic centre within the pores.

19. A method according to claim 1 or 13 characterised in that the particles have a mean size of 5000 nm or less.

20. A method according to claim 18 characterised in that the nanoparticles have a mean size of from 1 nm to 5000 nm.

21. A method according to claim 1 or 13 characterised in that the method comprises the application of a remote magnetic field on the magnetic particles.

22. A method according to claim 1 or 13 characterised in that the particle is tagged with one or more specific antibodies or protein binding motifs which recognise key cellular elements within a cell.

23. A method according to claim 22 characterised in that the specific antibodies or protein binding motifs are selected from transmembrane extracellular matrix molecules, adhesion molecules or dispersed membrane adhesion proteins or extracellular matrix proteins.

24. A method according to claim 23 characterised in that the specific antibodies or protein binding motifs are transmembrane adhesion or extracellular matrix molecules.

25. A method according to claim 24 characterised in that the transmembrane adhesion molecules are selected from integrins, cadherins, selectins, and immunoglobulins.

26. A method according to claim 23 characterised in that the specific antibodies or protein binding motifs are selected from dispersed membrane adhesion proteins.

27. A method according to claim 26 characterised in that the dispersed membrane adhesion protein is RGD (arginine-glycine-aspartate).

28. A method of treatment of a patient suffering from a disorder in which an ion channel plays a role which comprises the administration to such a patient of magnetic nanoparticles as hereinbefore described and manipulating the ion channels or cells using a magnetic field external to the body.

29. A method of treatment according to claim 28 in which a disorder may involve a number of tissues in the body where ion channels play a key role in normal cellular homeostasis.

30. A method according to claim 29 characterised in the cells are cardiac muscle cells.

31. A method according to claim 29 characterised in that the method comprises the treatment of hypertension.

32. A method according to claim 29 characterised in that the method comprises pain relief.

33. A method according to claim 32 characterised in that the method comprises anaesthesia.

34. A method according to claim 33 characterised in that the anaesthesia is localised.

35. A method of treatment of a patient according to claim 28 characterised in that the method comprises tissue and/or bone repair.

36. A method of treatment according to claim 35 characterised in that the cells are selected from ligamentum cells, tenocytes, chondrocytes and other stromal cells (such as chondrocyte progenitor cells).

37. A method of treatment according to claim 35 characterised in that the method comprises the regeneration of tissue or the generation of artificial tissue, such as skin, cartilage, ligament, tendon, muscle or bone.

38. A method of treatment according to claim 35 characterised in that the method comprises wound healing and/or tissue adhesion.

39. A method of treatment according to claim 35 characterised in that the method comprises bone repair and/or bone growth.

40. A method of treatment according to claim 28 characterised in that the method comprises a dental or veterinary application.

41. A method for establishing localised anaesthesia through the action of ion channel modulation by magnetic fields external to the body.

42. A method according to claim 41 characterised in that the pain relief comprises anaesthesia.

43. A method of treatment according to claim 28 characterised in that the method comprises the use of a magnetic field at a frequency of from 0.1 to 10 Hz.

44. A method of treatment according to claim 28 characterised in that the method comprises the use of a magnetic field will typically have a flux density of from 10 mT to 1400 mT.

45. The use of a magnetic nanoparticle in a method of magnetically remotely manipulating cells *in vivo*.

46. The use according to claim 45 characterised in that the use comprises manipulating cells from outside the body.

47. The use according to claim 45 characterised in that the use comprises a method of tissue repair and/or bone repair.

48. The use according to claim 45 characterised in that the use comprises a method of pain relief.

49. The use according to claim 48 characterised in that the pain relief comprises anaesthesia.

50. A method substantially as described with reference to the accompanying drawings.

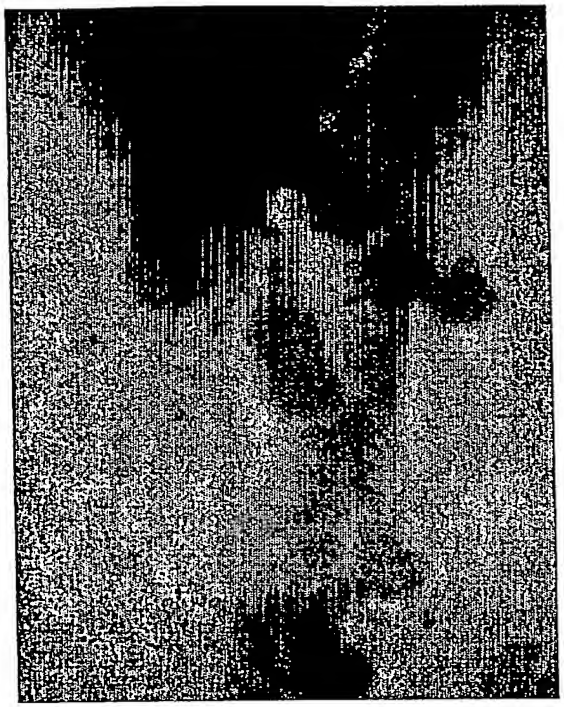

Figure 1b

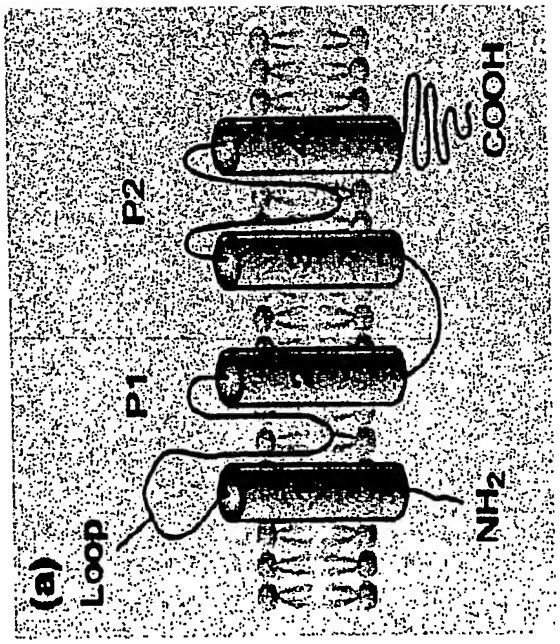

Figure 1a